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(54) Title: PH GENES AND THEIR USES		
(57) Abstract		
<p>The present invention provides compositions and methods for regulating vacuolar pH. Isolated DNA constructs comprising sequences substantially identical to a <i>Ph</i> gene are provided. The methods typically involve introducing the construct into a plant, whereby vacuolar pH is modified in the transgenic plant.</p>		

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PH Genes and Their Uses

The present application is a continuation in part of
5 USSN 08/049,282, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to compositions and
methods for controlling intra-cellular pH, e.g., vacuolar pH
in plant cells. In particular, it relates to the isolation
10 and characterization of *Ph* genes and their use in controlling
vacuolar pH.

The vacuole is an important component of plant cells
and often accounts for the majority of the cell volume. The
vacuole contains water and a variety of organic and inorganic
15 compounds such as sugars, organic acids, proteins, anthocyanin
pigments and excretory products such as calcium oxalate and
tannin compounds.

The vacuole is surrounded by a membrane (tonoplast
membrane) which regulates the movement of materials into and
20 out of the vacuole. Thus, the plant cell controls the
contents of the fluid or cell sap within the vacuole. One of
the properties of the cell sap which is regulated by the plant
cell is proton concentration or pH of the solution.

A number of proteins can affect vacuolar pH. For
25 instance, vacuolar ATPases generate pH and electrical
gradients by hydrolyzing ATP and pumping protons across the
tonoplast membrane. Nelson et al., *Trends in Biochemical
Science*, 14:113-116 (1989). Another enzyme that affects
vacuolar pH is inorganic pyrophosphatase. In *Arabidopsis*,
30 this enzyme is a single 81 kDa protein that is encoded by a
single gene (Sarafian et al., *Proc. Natl. Acad. Sci. USA*
89:1775-1779 (1992)).

A group of genes in plants, referred to as *Ph* genes,
encode proteins that also affect vacuolar pH. In *Petunia*
35 *hybrida* (petunia) a number of *Ph* genes are known to play a
role in determining vacuolar pH, although none has been
reported as isolated or sequenced. de Vlaming et al. *Theor.*
Appl. Genet. 66:271-278 (1983) and Viering, et al., in

Monographs on Theoretical and Applied Genetics 9: Petunia, K.C. Sink, ed. (Springer-Verlag, Berlin 1984), pp. 49-67, Gerats et al., *Dev. Gen.* 10:561-568 (1989) all of which are incorporated herein by reference.

5 In yeast, at least 17 different *vph* (vacuolar pH genes) required for acidification have been identified. For example, the *vph1* mutation causes an elevation of vacuolar pH. Preston et al., *Proc. Natl. Acad. Sci. USA* 86:7027-7031 (1989). This mutant has been shown to lack ATPase activity as
10 a consequence of a lesion in a gene encoding a 95kDa integral membrane subunit of the ATPase. Manolson et al. *J. Biol. Chem.* 267:14294-14303 (1992). A second *vph* mutant, *vat2*, is defective in the synthesis of the 60 kDa subunit of ATPase and also exhibits an elevated vacuolar pH. Nelson et al. *Proc.*
15 *Natl. Acad. Sci. USA* 87:3503-3507 (1990).

Vacuolar pH is important in determining a number of plant traits. For instance, in petunia, certain forms of anthocyanins have been shown to appear red at low pH (e.g., pH 5.5) and blue at higher pH values (e.g., pH 5.9). Timberlake
20 et al., in *The Flavonoids*, Harborne, et al. eds. (Academic Press, New York, 1975) pp.214-266. Thus, flower color has been shown to change as the pH of the corolla cell vacuole is altered. In addition, the acidity of fruits such as tomatoes or citrus fruits depends upon the pH of the vacuolar contents.
25 Other traits known to be affected by vacuolar pH include seed coat development, female fertility, protein transport.

There is currently a need for methods of producing new plant varieties with modified traits affected by vacuolar pH, such as flower color and fruit acidity. Control of the
30 expression of genes encoding proteins affecting vacuolar pH provides a useful approach to this problem. The present invention addresses these and other needs.

SUMMARY OF THE INVENTION

35 The present invention provides isolated DNA constructs comprising a polynucleotide sequence from a *Ph* gene. As defined here, *Ph* genes encode proteins which regulate vacuolar pH. Preferably, the *Ph* gene regulates pH by

controlling transcription of one or more genes (i.e., downstream genes) which in turn encode proteins which directly regulate vacuolar pH. The protein encoded by such *Ph* genes typically comprises a transcriptional activator sequence in particular, a helix-loop-helix motif. This motif is characteristic of the *myc* family of eukaryotic transcriptional activators. A preferred embodiment of pH gene comprises sequences substantially identical to sequences which are, or which are contained within SEQ. ID. No. 1.

Also within the invention are genes the mutation of which confer one or more of the following characteristics on a plant compared to wild type: 1) increased vacuolar pH; 2) flower color shift from red toward blue as a result of pH effect on anthocyanin pigments; 3) enhanced fading of flower color with aging; and 4) appearance of seeds as shrivelled or irregular and having less pigment.

The constructs of the invention are used to alter the vacuolar pH of plant organs or parts such as flowers or fruit, typically by modifying expression of an endogenous *Ph* gene. Thus, the DNA construct may further comprise a promoter operably linked to the polynucleotide sequence. The promoter is preferably a plant promoter such as a fruit-specific promoter or a flower-specific promoter. If suppression of the endogenous *Ph* gene is desired, the polynucleotide sequence may be linked to the promoter in the sense or antisense orientations.

The invention also provides transgenic plants (e.g., petunia plants or tomato plants) comprising a recombinant expression cassette which includes a plant promoter operably linked to the polynucleotide sequence. The transgenic plants exhibit altered vacuolar pH in one or more types of tissues. For many purposes, the introduction of the recombinant expression cassettes preferably results in inhibition of an endogenous *Ph* gene, resulting in plants with increased vacuolar pH.

The invention further provides a method of altering vacuolar pH in a plant. The method comprises introducing into plant tissue a recombinant expression cassette comprising a

plant promoter operably linked to a polynucleotide sequence from a *Ph* gene, in the sense or the antisense orientation. The promoter may be a tissue-specific promoter, e.g., a fruit-specific promoter or a flower-specific promoter. The expression cassette is typically introduced into the plant tissue using *Agrobacterium* or other standard means. The transformed plant tissue is regenerated into whole plants, whereby normally the regenerated plant transcribes the introduced polynucleotide sequence. The plants are then assayed and selected for altered vacuolar pH.

The invention further provides methods of isolating a *Ph* gene from a plant. The method may comprise probing a DNA library (e.g., a cDNA library) prepared from the plant with oligonucleotide probes comprising a polynucleotide sequence from an isolated *Ph* gene. Alternatively, the method may comprise transforming plants with a DNA construct comprising a transposon (e.g., the *Ac* transposon) and assaying the plants for increased vacuolar pH resulting from the transposon excising from the DNA construct and inserting in a *Ph* gene. Those plants having increased vacuolar pH are then selected. A preferred method uses a transposon inserted in a streptomycin resistance gene such that the gene is inoperable. Plants in which the transposon has excised from the gene are identified by the ability to grow on streptomycin.

25

Definitions

The phrase "nucleic acid sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA and non-functional DNA or RNA.

The term "promoter" refers to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells.

The term "plant" includes whole plants, plant organs (e.g., leaves, stems, roots, tc.), seeds and plant cells and

progeny of same. The class of plants which can be used in the method of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. It includes
5 plants of a variety of ploidy levels, including polyploid, diploid and haploid.

A "heterologous sequence" is one that originates from a foreign species, or, if from the same species, is substantially modified from its original form. For example, a
10 heterologous promoter operably linked to structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, is substantially modified from its original form.

A "polynucleotide sequence from a *Ph* gene" is a
15 subsequence or full length polynucleotide sequence of a *Ph* gene, such as the *Ph6* gene, which, when present in a transgenic plant has the desired effect, for example, inhibiting expression of the endogenous *Ph* gene. In the case of both expression of transgenes and inhibition of endogenous
20 genes (e.g., by antisense, or sense suppression) one of skill will recognize that the inserted polynucleotide sequence need not be identical and may be "substantially identical" to a sequence of the gene from which it was derived. As explained below, these variants are specifically covered by this term.

25 In the case where the inserted polynucleotide sequence is transcribed and translated to produce a functional polypeptide, one of skill will recognize that because of codon degeneracy a number of polynucleotide sequences will encode the same polypeptide. These variants are specifically covered
30 by the above term. In addition, the term "polynucleotide sequence from a *Ph* gene" specifically includes those full length sequences substantially identical (determined as described below) with a *Ph* gene sequence and that encode proteins that retain the function of the *Ph* protein. Thus, in
35 the case of *Ph6* gene disclosed here, the above term includes variant polynucleotide sequences which have substantial identity with the sequences disclosed here and which encode

proteins capable of altering vacuolar pH as detected in the assays described below.

In the case of polynucleotides used to inhibit expression of an endogenous gene, the introduced sequence also need not be perfectly identical to a sequence of the target endogenous gene. The introduced polynucleotide sequence will typically be at least substantially identical (as determined below) to the target endogenous sequence.

Two nucleic acid sequences or polypeptides are said to be "identical" if the sequence of nucleotides or amino acid residues, respectively, in the two sequences is the same when aligned for maximum correspondence as described below. The term "complementary to" is used herein to mean that the complementary sequence is identical to all or a portion of a reference polynucleotide sequence.

Sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two sequences over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman and Wunsch *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson and Lipman *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection. These references are incorporated herein by reference.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison

window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 60% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using the programs described above (preferably BESTFIT) using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 40%, preferably at least 60%, more preferably at least 90%, and most preferably at least 95%.

In the case of *Ph* genes and proteins of the invention, sequences are preferably compared in regions outside the conserved helix-loop-helix region characteristic of transcriptional activators as described below. Unrelated transcriptional activators may have high sequence identity only in the helix-loop-helix region, whereas *Ph* genes of the invention are substantially identical to the sequences disclosed here in the helix-loop-helix region as well as sequences outside this region.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Stringent conditions are sequence dependent and will be different in different

circumstances. Generally, stringent conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C.

As used herein a "Ph gene" is a gene encoding a protein, other than an ATPase or pyrophosphatase, that regulates or affects vacuolar pH. Ph genes typically affect a number of plant characteristics including flower color, stability of flower color over time, fertility and seed appearance.

A Ph gene may be identified and defined based upon the fact that when normal Ph gene expression is disrupted (e.g., because of the presence of a mutant Ph gene) the plants exhibit one or more of the following characteristics: 1) increased vacuolar pH as determined using the assays described below; 2) flower color shift from red toward blue as a result of pH effect on anthocyanin pigments; 3) enhanced fading of flower color with aging; and 4) appearance of seeds as shrivelled or irregularly shaped and less pigmented than wild-type. Such characteristics may also result from suppression of the Ph gene using suppression methods taught herein. In addition, allelism tests can be used to determine whether the mutant gene is capable of complementing a known Ph gene.

The Ph genes of the invention preferably encode proteins which affect the transcription of one or more genes encoding proteins affecting vacuolar pH. Typically, a Ph gene will encode a protein which is a transcriptional activator having a helix-loop-helix motif, as described below.

As used herein, a homolog of a particular Ph gene (e.g. the petunia Ph6 gene) is a second gene (either in the same plant type or in a different plant type) which has a polynucleotide sequence of at least 50 contiguous nucleotides which are substantially identical (determined as described

above) to a sequence in the first gene. It is believed that, in general, homologs share a common evolutionary past.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1A shows a variegated petunia corolla produced by a plant homozygous for the *ph6-m1(Ac)* allele.

 Figure 1B shows a plant with a variegated mutant flower (bottom) and solidly colored revertant flower (top).

10 Figure 1C shows a branch showing an older variegated flower (left) with a faded background color relative to that of the recently opened variegated flower (right).

 Figures 2A and 2B show DNA gel blot analysis of variegated and solidly colored progeny of plant 3057.12, carrying a *trAc* element. P indicates the 3057.12 parent; V1 to V6, variegated progeny; S1 to S8, solidly colored progeny; GB, nontransgenic plant of V26 genetic background. Molecular length markers are given at left in kilobases. Figure 2A shows hybridization to a probe made from the *EcoRI*-*HindIII* fragment of *Ac*. Figure 2B shows hybridization to a probe made from the *BstXI*-*EcoRI* fragment flanking the *Ac* insertion.

20 Figure 2C shows a restriction map of *Ph6* DNA mutated by insertion of *Ac*. The positions of the probes corresponding to *Ac* and to the flanking DNA are indicated by bars above the map.

25 Figures 3A and 3B are DNA gel blot analyses of revertants. The blot was probed with the *BstXI*-*EcoRI* fragment from DNA flanking the *Ac* insertion (bar in Figure 2C). Molecular length markers are given at left in kilobases. Figure 2A shows analysis of the somatic sector shown in Figure 1B. Lane 1, 3057.12 parent plant; lanes 2 and 3, leaf and flower, respectively, borne on a variegated branch; lanes 4 and 5, leaf and flower, respectively, borne on a solidly colored branch. Figure 3B is germinal revertants. Lane 1, V26 inbred line; lanes 2, 5, and 6, solidly colored progeny of a variegated plant; lanes 3 and 4, variegated progeny of same plant.

35 Figure 4 shows seeds produced by the *ph6-m1(Ac)* mutation. The pigmented seed indicated by the arrow looks

normal and probably arose as a consequence of an *Ac* excision event early in seed coat development.

Figures 5A and 5B show flower phenotypes from the allelism test to *ph6*. Figure 5A shows *Ph6/ph6* (top) and *ph6-m1(Ac)/ph6* (bottom), both in a V26/W160 genetic background. Figure 5B is a magnification of bottom flower in 5A showing the small revertant sectors on a mutant background.

Figure 6A-C is a RNA gel blot of wild-type and mutant flower buds and leaves hybridized sequentially to three probes. Figure 6A shows hybridization to the *EcoRI*-*BamHI* DNA fragment on the left side of the *Ac* insertion (Figure 2C). Figure 6B shows hybridization to the flower-specific *CHS-A* probe. Figure 6C shows hybridization to a wheat rDNA probe. Lane 1, mutant leaf; lane 2, 2-cm mutant flower bud; lane 3, wild-type leaf; lane 4, 2-cm wild-type flower bud. Top arrow indicates the position of 28S rRNA; bottom arrow, position of 18S rRNA.

Figure 7 is a schematic representation of two recombinant constructs of the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions and methods for regulating vacuolar pH. The methods of the present invention may employ recombinant vectors comprising polynucleotide sequences from a *Ph* gene as described below. *Ph* genes useful in the present invention include *Ph* genes identified in petunia, as well as homologs in petunia and other plants (either of the same or different genus or species).

The effect of *Ph* genes on vacuolar pH has been elucidated by studying homozygous recessive alleles of these genes. For instance, in plants homozygous for the recessive allele of either the *Ph1* or *Ph2* gene (designated as *ph1* and *ph2*) corolla pH is increased and the flowers have bluish color. In plants in which the *ph3* gene is homozygous, corolla pH is increased, and female sterility is exhibited. If the *ph4* gene is homozygous, the effects are similar to those of *ph3*, but the plants are fertile. Viering et al., *supra*.

The *Ph* genes may also be associated with other flower color traits. For instance, it has been observed that flower color fades in some plants homozygous recessive for *ph3* and *ph4*. Typically, about two to three days after the flower opens, anthocyanin begins to disappear and the flowers are completely white by the time they wither. Genetic experiments have shown that the fading trait is restricted to plants accumulating certain classes of anthocyanins. de Vlaming et al. *Theor. Appl. Genet.* 61:41-46 (1982).

The amino acid sequence in SEQ. ID. No. 2, as shown, comprises a helix-loop-helix structural motif starting with the sequence NHVLAER (starting at residue 191) and extending to the sequence KKVQDLE (ending at residue 244). Helix-loop-helix motifs are found in a number of eukaryotic genes and are thought to be involved in DNA binding during transcriptional activation. Proteins containing this motif include the *myc* family of oncogenes, regulators of neuron and muscle development, and regulators of segmentation and organ patterning in *Drosophila*. In plants, the motif is found in *R(S)*, a protein involved with regulating anthocyanin synthesis in maize. Ludwig et al. *Proc. Natl. Acad. Sci. USA* 86:7092-7096 (1989). A comparison of the *Ph6* and *R(S)* sequences over the 54 amino acid helix-loop-helix region performed by the BESTFIT program of GCG using standard parameters (gap weight: 3.000, length weight 0.100) detected 57% amino acid identity between the two proteins. Outside this region only 19% identity was found.

Without wishing to be bound by any particular theory, it is believed that, unlike previously described proteins encoded by transcriptional activators, the proteins encoded by the *Ph* genes of the invention specifically target a downstream gene or genes encoding proteins which control vacuolar *Ph*. That is, unlike ATPases and pyrophosphatases, the *Ph* proteins modulate vacuolar pH by regulating the expression of other proteins that directly affect vacuolar pH. Thus, the methods of the invention allow the control of vacuolar pH by suppressing or enhancing the expression of a

single *Ph* gene, which in turn regulates the expression of one or more downstream genes.

Using the methods of the present invention vacuolar pH can be adjusted as desired. For instance, vacuolar pH may be increased or decreased by up to 1 pH unit or more, typically about 0.5 pH unit. Changes of 0.3 pH unit or less (e.g., 0.2 or 0.1 pH unit) may be desirably obtained. The changes in pH can be detected using the assays described below.

Any plant trait affected by vacuolar pH can be modified using the methods of the invention. Such modification may involve flowers, fruits or other plant parts. For instance, flower color of ornamental plants such as petunias, roses, carnations and the like can be modified. Storage properties of the fruit can also be improved by adjusting pH. Fruit acidity can be adjusted in fruits of various plants, e.g., citrus, tomato, grape, pineapple, tropical fruits (mango, passion fruit, papaya), berries (e.g., strawberry), melon, bananas, apples, pear, peach, apricot, nectarine, cherry, avocado, kiwi, and coffee. Also, the pH of other plant tissues, e.g. edible vegetable parts, can be modulated using these methods.

In general, the flavor of fruits depends upon the interaction of four classes of compounds: sugars, acids, non-volatile flavor compounds and volatile "aroma" compounds. The ratio of sugar:acid, in combination with specific flavor and aroma compounds, gives the unique flavor of any specific fruit. Flavor intensity is often determined more by the absolute levels of sugars and acids than by individual flavor compounds.

The flavor of many fruits, e.g., tomato or citrus fruits, is determined, in part, by acidity of cells of the fruit. The balance of sweetness and sourness is key to the flavor of a wide range of fruits, with the relative acidity of fruit determining the relative sourness. This has been shown by Stevens et al., *J. Amer. Soc. Hort. Sci.*, 104:40-42, (1979) in comparing a set of tomato lines. See also Sakiyama et al., *J. Amer. Soc. Hort. Sci.*, 101:394-96, (1976), and Picha,

HortScience, 22:94-96, (1987) regarding tomato acidity. Stevens et al. also demonstrated that increasing acidity contributes to increasing intensity of tomato flavor. In tomato fruit, a range of pH values, from 4.0 to 5.0, is found among tomato varieties. In general, a lower fruit pH within that range gives better flavor intensity. The invention may be used to adjust fruit pH within known ranges or to move it outside those ranges.

The invention has use in altering pH-related traits in all higher plants, e.g., flavor (if edible), color or both. The invention thus has use over a broad range of types of plants, including species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Hyoscyarnus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Ciahorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hererocallis*, *Nemesis*, *Pelargonium*, *Panieum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*, and *Datura*.

More specifically, plants for which the invention may be used in modifying acidity-related traits (flower, color or otherwise) includes oil crops such as canola (*Brassica* sp.), cotton (*Gossypium* sp.), peanut (*Arachis* sp.), sunflower (*Helianthus* sp.), palm (*Elaeis* sp.), flax (*Linum* sp.), safflower (*Carthamus* sp.), coconut (*Cocos* sp.) and soybean (*Glycine* sp.); grain crops such as wheat (*Triticum* sp.), corn (*Zea* sp.), sorghum (*Sorghum* sp.), barley (*Hordeum* sp.), rye (*Secale* sp.), oats (*Avena* sp.) and rice (*Oryza* sp.); fruit crops such as banana (*Musa* sp.), citrus (*Citrus* sp.), berries (e.g., strawberry (*Fragaria* Sp.) or raspberry (*Rubus* sp.), mango (*Mangifera* sp.), melon (*Cucumis* sp.), pear (*Pyrus* sp.), cucumber (*Cucumis* sp.), and apricot, peach, cherry, plum and prune (*Prunus* sp.); vegetable crops such as pea (*Pisum* sp.), bean (*Vicia* sp.), broccoli and related crucifers (*Brassica* sp.), spinach (*spinacia* sp.), onion (*Allium* sp.), celery (*Apium* sp.), carrot (*Daucus* sp.), asparagus (*Asparagus* sp.),

and artichoke (*Helianthus* sp.); additional ornamental crops such as tulip (*Tulipa* sp.), snapdragon (*Antirrhinum* sp.), Iris (*Iris* sp.), Orchids (*Cymbidium* and *Cattleya* sp.), pelargonium; beverage crops such as coffee (*Coffea* sp.) and tea (*Thea* sp.);
5 herb crops such as mint (*Mentha* sp.), thyme (*Thymus* sp.) and marjoram (*origanum* sp.).

The control of the expression of genes associated with vacuolar pH, in particular *Ph* genes, can be achieved by introducing mutations into the gene or using recombinant DNA
10 techniques. These techniques are generally well known to one of skill and are discussed briefly below.

Using the methods described below, the plants having the desired mutation (e.g., inhibited *Ph* gene expression) can be selected by assaying for changes in vacuolar pH. Thus,
15 where the goal is inhibition of *Ph* gene expression plants having increased vacuolar pH are selected. Where overexpression of the *Ph* gene is desired plants having decreased vacuolar pH are selected. Alternatively, other desired phenotypic changes such as changes in pigmentation, as
20 evidenced by e.g., modified flower color, can be used.

A number of methods are useful for introducing a genetic mutations into a *Ph* gene. For instance, seeds or other plant material can be treated with a mutagenic chemical substance, according to standard techniques. Such chemical
25 substances include, but are not limited to, the following: diethyl sulfate, ethylene imine, ethyl methanesulfonate and N-nitroso-N-ethylurea. Alternatively, ionizing radiation from sources such as, for example, X-rays or gamma rays can be used. Mutant plants or their progeny are then selected based
30 on the desired altered phenotypes (resulting from mutations in *Ph* genes), such as flower color, fruit acidity, or pH of homogenized tissues using the assays described below. Mutation of the transcriptional activator sequence of a *Ph6* gene can result in suppression of the downstream genes
35 controlled by the *Ph6* gene; Goff et al., *Genes & Dev*, 5:298-309 (1991).

The Example section below, which describes the isolation and characterization of the *Ph6* gene in Petunia, is

exemplary of a general approach for isolating Ph genes. Isolation of this gene allows one of skill to readily isolate homologous genes in Petunia and other plant species. The isolated genes can then be used to construct recombinant
5 vectors for altering Ph gene expression in transgenic plants.

Generally, the nomenclature and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation,
10 amplification and purification. Generally enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturer's specifications. These techniques and various other techniques are generally performed according to Sambrook
15 et al., *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989), which is incorporated herein by reference.

The isolation of Ph genes may be accomplished by a number of techniques. For instance, transposon tagging of a
20 Ph gene can assist in the isolation of the relevant gene. Transposon tagging involves introducing a transposon into the plant which leads to a mutation of the target gene and a detectable phenotypic change in the plant. Using a probe for the transposon, the mutant gene can then be isolated. Using
25 the DNA adjacent to the transposon in the isolated mutant gene as a probe, the normal wild type allele of the target gene can be isolated. See, e.g., Haring, et al., *Plant Mol. Biol.* 16:449-469 (1991) and Walbot, *Ann. Rev. Plant Mol. Biol.* 43:49-82 (1992). As shown below, a particularly useful
30 transposon tagging system is that disclosed in U.S. Patent No. 5,013,658, which is incorporated herein by reference.

An alternative method uses oligonucleotide probes to identify the desired gene in a cDNA or genomic DNA library. To construct genomic libraries, large segments of genomic DNA
35 are generated by random fragmentation, e.g. using restriction endonucleases, and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. To prepare a cDNA library, mRNA is isolated from the desired

organ, such as a flower, and a cDNA library which contains the Ph gene transcript is prepared from the mRNA. Alternatively, cDNA may be prepared from mRNA extracted from other tissue types (organs) in which Ph genes or homologs are expressed such as seeds, fruits, leaves, stems, and roots.

The cDNA or genomic library can then be screened using a probe based upon the sequence of a cloned Ph gene such as Ph6. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. The use of such hybridization techniques for identifying homologous genes is well known in the art and need not be described further.

Alternatively, polynucleotides may be synthesized by well-known techniques as described in the technical literature. See, e.g., Carruthers et al., *Cold Spring Harbor Symp. Quant. Biol.* 47:411-418 (1982), and Adams et al., *J. Am. Chem. Soc.* 105:661 (1983), both of which are incorporated herein by reference. Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

The isolated sequences prepared as described herein, can be used in a number of techniques to suppress endogenous Ph gene expression (i.e., to raise the pH and thus lower acidity). For instance, antisense technology can be conveniently used to inhibit Ph gene expression. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the anti-sense strand of RNA will be transcribed. The construct is then transformed into plants and the anti-sense strand of RNA is produced. In plant cells, it has been shown that anti-sense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see, e.g., Sheehy et al., *Proc. Nat. Acad. Sci. USA*, 85:8805-8809 (1988), and Hiatt et al., U.S. Patent No. 4,801,340 which are incorporated herein by reference.

The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous *Ph* gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit
5 expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the target gene. For example, suppression of the *Ph6* gene may serve to impose the same
10 suppressive effect on other *Ph* genes with sufficient identity. Similarly, segments from *Ph* genes from *Petunia* can be used to inhibit expression of homologous genes in different plant species, e.g., using sense or antisense suppression techniques described herein either directly or as a means to obtain the
15 corresponding sequences to be used to suppress the gene.

The introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Furthermore,
20 the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Normally, a sequence of between about 30 or 40 nucleotides and about 2000 nucleotides should be used, though a sequence of at least about 100 nucleotides is preferred, a
25 sequence of at least about 200 nucleotides is more preferred, and a sequence of at least about 500 nucleotides is especially preferred.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of *Ph* genes. It is possible to
30 design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and
35 cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs which are capable of self-cleavage and replication in plants. The RNAs replicate either alone
5 (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, solanum nodiflorum mottle virus and subterranean clover mottle virus. The design
10 and use of target RNA-specific ribozymes is described in Haseloff et al. *Nature*, 334:585-591 (1988), which is incorporated herein by reference.

Another method of suppression is sense suppression. Introduction of nucleic acid configured in the sense
15 orientation has been recently shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli et al., *The Plant Cell* 2:279-289 (1990), and U.S. Patent No. 5,034,323, which are incorporated
20 herein by reference.

Generally, where inhibition of expression is desired, some transcription of the introduced sequence must occur. The effect may occur where the introduced sequence contains no coding sequence *per se*, but only intron or
25 untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a
30 higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 80% is preferred, though about 95% to absolute identity would be most preferred. As with
35 antisense regulation, the effect should apply to any other proteins within a similar family of genes exhibiting homology or substantial homology.

The introduced sequence, needing less than absolute identity, also need not be full length, relative to either the

primary transcription product or fully processed mRNA. A higher identity in a shorter than full length sequence compensates for a longer, less identical sequence.

Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments will be equally effective. Normally, a sequence of the size ranges noted above for antisense regulation is used.

Isolated sequences prepared as described herein can also be used to enhance or increase endogenous Ph gene expression (i.e., to lower the pH and thus raise acidity). Where overexpression of the Ph gene is desired, a Ph gene from a different species may be used to decrease potential sense suppression effects. For instance, the petunia Ph6 gene can be used to increase expression in tomato fruit.

One of skill will recognize that the polypeptides encoded by the Ph genes, like other proteins, have different domains which perform different functions. Thus, the Ph gene sequences need not be full length, so long as the desired functional domain of the protein is expressed. Modified protein chains can also be readily designed utilizing various recombinant DNA techniques well known to those skilled in the art and described in detail, below. For example, the chains can vary from the naturally occurring sequence at the primary structure level by amino acid substitutions, additions, deletions, and the like. These modifications can be used in a number of combinations to produce the final modified protein chain.

To use isolated Ph sequences in the above techniques, recombinant DNA vectors suitable for transformation of plant cells are prepared. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, for example, Weising et al. *Ann. Rev. Genet.* 22:421-477 (1988), which is incorporated herein by reference. A DNA sequence coding for the desired Ph polypeptide, for example a cDNA sequence encoding a full length protein, will be combined with transcriptional and translational initiation regulatory sequences which will direct the transcription of

the sequence from the *Ph* gene in the intended tissues of the transformed plant.

For example, a plant promoter fragment may be employed which will direct expression of the *Ph* in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, and other transcription initiation regions from various plant genes known to those of skill.

Alternatively, the plant promoter may direct expression of the *Ph* gene in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here as "inducible" promoters. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions or the presence of light.

Examples of promoters under developmental control include promoters that initiate transcription only in certain tissues, such as fruit, seeds, or flowers. For example, the use of a polygalacturonase promoter can direct expression of the *Ph* polypeptide in the fruit, a CHS-A (chalcone synthase A from petunia) promoter can direct expression of the *Ph* polypeptide in flower of a plant.

If proper polypeptide expression is desired, a polyadenylation region at the 3'-end of the *Ph* coding region should be included. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA.

The vector comprising the sequences from a *Ph* gene will typically comprise a marker gene which confers a selectable phenotype on plant cells. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin,

hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

Such DNA constructs may be introduced into the genome of the desired plant host by a variety of conventional techniques. For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation and microinjection of plant cell protoplasts, or the DNA constructs can be introduced directly to plant tissue using ballistic methods, such as DNA particle bombardment. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria.

Microinjection techniques are known in the art and well described in the scientific and patent literature. The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski et al. *Embo J.* 3:2717-2722 (1984). Electroporation techniques are described in Fromm et al. *Proc. Natl. Acad. Sci. USA* 82:5824 (1985). Ballistic transformation techniques are described in Klein et al. *Nature* 327:70-73 (1987). The full disclosures of each of these references are incorporated herein by reference.

Agrobacterium tumefaciens-mediated transformation techniques are well described in the scientific literature. See, for example Horsch et al. *Science* 233:496-498 (1984), and Fraley et al. *Proc. Natl. Acad. Sci. USA* 80:4803 (1983), all of which are incorporated herein by reference.

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype and thus the desired *Ph*-controlled phenotype. Such regeneration techniques rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with the *Ph* nucleotide sequences. Plant

regeneration from cultured protoplasts is described in Evans et al., *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, pp. 124-176, MacMillilan Publishing Company, New York, 1983; and Binding, *Regeneration of Plants, Plant Protoplasts*, pp. 21-73, CRC Press, Boca Raton, 1985, all of which are incorporated herein by reference. Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee et al. *Ann. Rev. of Plant Phys.* 38:467-486 (1987); which is incorporated herein by reference.

The methods of the present invention are particularly useful for incorporating the *Ph* genes into transformed plants in ways and under circumstances which are not found naturally. In particular, the *Ph* polypeptides may be expressed at times or in quantities which are not characteristic of natural plants.

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

The effect of the modification of *Ph* gene expression is conveniently detected by measuring vacuolar pH. Vacuolar pH can be measured using conventional assay techniques. For example, corolla pH can be detected by using pH meter to measure pH in fresh flower tissue homogenized in water as described by de Vlaming et al., *supra*.

In the case of desired modifications to flower color, inhibition of *Ph* gene expression can be detected by a change in anthocyanin pigmentation from red to blue. In addition, antisense or sense suppression of the endogenous gene can be detected by reduction of mRNA levels as measured by, for instance, Northern blots.

The following Examples are offered by way of illustration, not limitation.

Examples

I. Isolation of a Variegated Flower Color Mutation in a Petunia Line Carrying Ac

This Example describes the generation of independent transformants carrying the Ac transposable element in Petunia. The stain used here, V26, a highly inbred, purple-flowered genetic line was obtained from the collection at the Free University of Amsterdam.

The binary vector used in transformation, pJJ4411, was constructed generally as described in U.S. Patent No. 5,013,658. The vector was constructed as described in Keller et al., *Plant Mol. Biol.* 21:157-170 (1993), which is incorporated herein by reference.

In addition to a hygromycin resistance transformation marker, this vector contains the streptomycin phosphotransferase (*SPT*::Ac excision marker between the right and left T-DNA borders. The maize element Ac interrupts the *SPT* gene and prevents its expression. In several plants, such as tobacco and Arabidopsis, this marker is a useful visual indicator of somatic and germinal Ac activity. In petunia, however, the streptomycin germination screen is not as reliable. It can be used to enrich for plants carrying transposed Ac elements (trAc). This procedure results in a greater than 10-fold enrichment for trAc in petunia.

One of the analyzed plants (3057.12) carried two Ac elements in heterozygous condition. One Ac element was still in its resident site in a T-DNA and the other at an unlinked chromosomal location, into which it had integrated following a secondary transposition event from a different T-DNA. Plant 3057.12 was selfed in order to make homozygous the transposed Ac (trAc) element and, therefore, any mutation caused by the trAc insertion.

When the self-progeny was planted, a new, variegated flower color phenotype was found to segregate as a simple recessive Mendelian trait. As can be seen in Figure 1A, the variegated flower phenotype is striking. In the flowers, darkly colored (revertant) sectors, outlined by white rims, stand out sharply against the pale colored (mutant) background of the corolla. The color of the background and the revertant

sectors varies depending on the residual genotype. In segregants from outcrosses to other genetic lines, the background color is blue and the revertant color red. In all flowers, a white rim separates the revertant sector from the mutant background.

Borders of different colors are rarely seen in examples of anthocyanin variegation. When they are seen, the rims tend to be more, not less, pigmented than the areas they delimit. The formation of new anthocyanin pigments in the border cells has been attributed to the diffusion of accumulated intermediates from adjacent cells. The presence of white rims in the variegated flowers of the plants of the present invention suggests that compounds can diffuse into the border cells from adjacent cells, i.e., that the effect of the mutated gene on anthocyanin pigmentation is not strictly cell autonomous.

II. Evidence That the New Mutation is Tagged by Ac

The following evidence indicates that the new variegated mutation has arisen as a consequence of an Ac transposition event, and was, therefore, tagged by Ac.

A. *Cosegregation with Ac-hybridizing band*

The mutation cosegregated with a new Ac-hybridizing band in DNA gel blots of the self progeny of plant 3057.12. DNA from the variegated (mutant) and solidly colored (parental) progeny was analyzed by digestion with different enzymes and hybridization with an Ac probe. To do this, genomic DNA (6 μ g) was digested with the enzyme, separated by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane according to standard techniques.

As seen in the genomic DNA gel blot shown in Figure 2A, in an EcoRI digest a new Ac-hybridizing band was found that cosegregated with the new variegated phenotype. In particular, a 4.7-kb band was present in every variegated plant (V1-V6), but only in some solidly colored siblings (S1, S2 and S3). The larger (5-kb) band represents the second unlinked Ac element. This band can be seen to segregate in both the variegated and the solidly colored siblings.

Among those variegated plants that received both Ac fragments, the intensity of the 4.7-kb band relative to the 5-kb band is either the same (V5) or double (V2, V3 and V4), suggesting that the new, 4.7-kb band is homozygous but that the 5-kb band can be either homozygous (V5) or heterozygous (V2, V3, V4). Conversely, some solidly colored progeny (S3 and S7) appear to be homozygous for the 5-kb, but not the 4.7-kb band.

In all, 26 variegated siblings were analyzed and all showed the new, Ac-hybridizing band at about the same relative intensity. Of 25 solidly colored siblings analyzed, 16 had the 4.7-kb band, a result in agreement with the proportion of heterozygotes for the new mutation (two thirds) expected within the solidly colored class. These data indicate that the new mutation is linked to the trAc band ($\chi^2=10.2$, $P<0.01$). Though no recombinant were found in the self progeny, the resolution of this type of F2 linkage data (repulsion phase, complete dominance) is limited, so the 95% confidence interval for the estimate of p , the recombination fraction, is large ($p=0$; CI= 0-0.34).

B. *Homozygosity for the Ac-tagged fragment*

All the mutant plants were homozygous for the Ac tagged DNA fragment. The Ac homologous, 4.7-kb EcoRI fragment, containing part of Ac and DNA adjacent to the Ac insertion, was cloned into the vector λ ZapII (Stratagene). A restriction map of this fragment (and of the adjacent 6.8-kb EcoRI fragment subsequently isolated) is shown in Figure 2C. The BstXI-EcoRI fragment flanking Ac in the 4.7-kb Ac fragment was labeled and used to reprobe the blot shown in Figure 2A. If the variegated plants are indeed homozygous for the new, 4.7-kb Ac band, they should lack the allelic wild-type fragment, which, conversely, should be present in all of the solidly colored siblings.

The DNA gel blot presented in Figure 2B confirms this. The solidly colored progeny show a 7-kb band of identical mobility to the band seen in the wild-type V26 inbred parent. In contrast, the variegated siblings either lack that band or show it at a very reduced intensity. The

weak band present in the variegated progeny can be attributed to occasional somatic excisions of *Ac*.

Those solidly colored progeny that did not show a 4.7-kb *Ac* homologous band in Figure 2A are homozygous for the V26 wild-type fragment. Solidly colored progeny that did show a 4.7-kb *Ac* homologous band are heterozygous for the V26 fragment.

The overall segregation obtained from having scored the progeny of plant 3057.12 with the two different probes (*Ac* and its flanking sequence) is as follows. Among the 25 solidly colored progeny analyzed, 16 were *Ac*/+, 9 were +/+, and none were *Ac*/*Ac*. Among the 26 variegated progeny, all were *Ac*/*Ac*. This more complete genotypic classification of the F2 progeny significantly reduces the size of the 95% confidence interval for *p* ($p=0$; CI= 0-0.05) and demonstrates that the new mutation is, in fact, closely linked to *Ac*.

C. Reversion to wild-type phenotype

Reversion of the mutation to the wild-type phenotype was correlated with restoration of a wild-type sized DNA fragment. Confirmation that a mutation is, in fact, tagged by *Ac* can be sought from an analysis of revertants because an excision of *Ac* that restores the wild-type phenotype should also produce a DNA fragment of the original wild-type size. Progeny from variegated plants were grown and screened for somatic and germinal reversion events. Branches with solidly colored flowers, representing large somatic revertant sectors, were occasionally seen on plants producing mostly variegated flowers (Figure 1B). DNA from the solidly colored and variegated branches of one such plant was prepared and analyzed by DNA gel blotting.

The blot shown in Figure 3A was probed with the BstXI-EcoRI DNA fragment flanking *Ac* (Figure 2C). Two bands of roughly equal intensity can be seen in the lanes containing DNA from a flower and a leaf that were borne on a revertant branch (lanes 4 and 5). One is a 7-kb, wild-type-sized band and the other, a 4.7-kb band, which also hybridizes to *Ac* (data not shown). This observation indicates that the revertant sectors are heterozygous for the original *Ac*-induced

mutation and a revertant allele produced by excision of *Ac* during development of the chimeric plant. Capsules borne on the revertant branches produced solidly colored and variegated individuals in a 3:1 ratio, confirming that the reversion event was heritable. The lanes containing DNA from a flower and leaf that were borne on a variegated branch (Figure 3A, lanes 2 and 3) show, in contrast, a strong 4.7-kb *Ac* band and a faint 7-kb band. The latter band probably represents empty sites generated by somatic excisions of *Ac* during the formation of the variegated flower. Capsules borne on variegated branches produced, as expected, mostly variegated progeny.

Plants with only solidly colored flowers were obtained among the progeny of variegated plants at frequencies ranging from 6 to 27%, indicating that the new mutation is also germinally unstable and reverts frequently to the wild-type state. Representative reversion data are shown in Table 1.

Table 1. Frequency of germinal revertants

Family	Pedigree	Number variegated plants	Number solid plants	Frequency germinal revertants ^a
3426	V26xM59	73	9	0.11
3428	V26xM59	103	25	0.19
3434	V26xM59	35	13	0.27
3466	V26xM59	51	9	0.15
3429	V26	22	2	0.08
3468	V26	91	6	0.06

^a Number of solidly colored plants to total number of plants.

Ten independent germinal revertants were analyzed by DNA gel blots, confirming the observations made earlier for the somatic revertant sectors. Figure 3B illustrates the analysis of three such germinal revertants. All the revertants (lanes 2, 5, and 6) showed the 7-kb, wild-type-sized band in addition to the 4.7-kb band. They are, therefore, heterozygous for a revertant allele and the

original *Ac*-induced mutation. The segregating variegated siblings (Figure 3B, lanes 3 and 4), on the other hand, showed only the 4.7-kb band; they are homozygous for the *Ac* mutation, consistent with their phenotype. In addition, six of eight
5 revertants analyzed had new *Ac* bands, indicating that the excised *Ac* elements continue to be capable of reinsertion.

The above evidence shows that the new variegated petunia mutant arose from the transposition of the maize element *Ac* into a gene affecting flower color.

10

III. The gene tagged by *Ac* affects the acidity of the corolla

Several considerations suggested that the new *Ac*-tagged mutant was a mutation of a *Ph* gene. First, in certain genetic backgrounds, the revertant sectors in the variegated
15 flowers appear red, whereas the mutant background has a distinct bluish hue. This color change is reminiscent of that brought about by the *Ph* mutations described above which cause a bluing of the corolla by increasing the vacuolar pH in the anthocyanin-accumulating cells (Viering et al., *supra*).
20 Anthocyanins in solution undergo a similar shift from red to blue as the acidity decreases.

Second, the new mutation affects the pH of the corolla in a manner similar to the known *Ph* mutant *ph1*. This was established by comparing the corolla pH of mutant and
25 revertant plants that arose in the self-progeny of a variegated plant, which was also *Hf1 Ph1/hf1 ph1*. *Hf1* and *Ph1* are closely linked genes on chromosome 1 that affect pigmentation of the corolla. *Hf1* controls hydroxylation at the 5' position of the anthocyanin B ring and causes a bluing
30 of the corolla. *Ph1* increases acidity in the vacuoles of the corolla and produces a more reddish hue.

The *Hf1 Ph1/hf1 ph1* heterozygote was obtained from an outcross of a variegated V26 plant (*Hf1 Ph1*) to line M59 (*hf1 ph1*). Among *Hf1* segregant, the pH of the corolla was
35 higher in the mutant plants (5.89 ± 0.02) than in the revertant plants (5.57 ± 0.03), a result that suggested that the new mutation altered the acidity of the corolla. This increase in pH can be compared to that caused by the *ph1* mutation in the

same segregating family. Because *ph1* and *hf1* are only 1 centimorgan apart, *hf1* segregant should also be *ph1/ph1* and, therefore, were used to compare the effects on corolla pH of *ph1* and of the new mutation. Two revertant progeny of the *hf1* class (and, presumably, *ph1/ph1*) were recovered, and both had high corolla pH values (5.90 ± 0.05), similar to those measured in the variegated flowers of the *Hf1* class.

Third, in some variegated plants the color of the flower fades with aging (Figure 1C), a phenomenon that has also been observed in petunia lines carrying certain *ph* mutations in conjunction with the *Fa* allele as described above (de Vlaming et al., *supra*). The pigments present in the faded corollas of the variegated line were compared with those present in the faded corollas of a *ph4* mutant line. In both cases, the faded flowers accumulated a phenolic compound fluorescing blue under ultraviolet light. This compound was absent in extracts of recently opened, nonfaded, flowers.

Fourth, the new mutation has a pleiotropic effect on seed development, an effect that has also been associated with some *ph* mutations (Viering, *supra*). When examined under 30x magnification, seeds borne on mutant plants appear abnormal. Some are shrivelled or irregularly shaped and the vast majority are variegated. The seed coat of normal petunia seeds is uniformly pigmented and reticulated. As shown in Figure 4, the mutant seed coat is largely unpigmented and lacks the honeycomb network of normal seeds, except for areas of varying size where the pigmented and reticulated peripheral structure is restored. The variegated seed phenotype of the *Ac*-induced mutation can be readily explained as another manifestation of somatic instability: the normal sectors on the mutant seed coats would form as a result of *Ac* excisions that occurred during seed development.

The observations given above cumulatively suggested that *Ac* had become inserted in one of the *Ph* genes. Allelism tests were conducted with known *ph* mutants to establish that the variegated mutant was capable of complementing all the mutants tested, except for *ph6*, as illustrated in Figure 5. The new, *Ac*-tagged *ph6* mutation was designated *ph6-m1(Ac)*.

The petunia line carrying the standard *ph6* mutation used in the complementation test is W160. (Available from the collection at the Free Univ. of Amsterdam).

5 The flower phenotype conditioned by the *ph6-m1(Ac)* allele in outcrosses to W160 is clearly different from that produced in the pure V26 line or in a mixed V26/M59 genetic background. Only small sectors can be seen (Figure 5) due to reversion events that occur late in flower development. The seed phenotype is similarly affected: a few of the seeds borne
10 on the outcross plants show traces of pigmentation, but the majority are unpigmented.

IV. The *ph6-m1(Ac)* mutation encodes an altered form of a flower-specific transcript

15 To detect a *Ph6* transcript, total RNA was prepared from *Ph6* and *ph6-m1(Ac)* flower buds and leaves. The RNAs were separated on a 1.1% agarose gel, blotted onto a nylon membrane, and probed with the EcoRI-BamHI fragment that extends from the BamHI site in *Ac* to the left of the insertion
20 site (Figure 2C). The corresponding RNA gel blot is shown in Figure 6A. An approximately 2.8-kb transcript was detected in wild-type flower buds (lane 4). In the mutant flower bud (lane 2), only a trace of the 2.8-kb transcript can be seen; the major signal is given, instead, by a 2-kb transcript.
25 Possibly, alternate splicing caused by the *Ac* insertion accounts for the multiple transcripts seen in the mutant (Wessler, 1988). No transcript was detected in either mutant or wild-type leaves (lanes 1 and 3), suggesting that the *Ph6* gene is expressed preferentially in flowers.

30 The RNA gel blot was rehybridized with the petunia flower-specific chalcone synthase probe *CHS-A* (Koes et al., *Plant Mol. Biol.* 12:213-225 (1989)) after washing away the first probe (Figure 6B). Transcripts of the same size and intensity were detected in mutant and wild-type flower buds
35 (lanes 2 and 4, respectively), but not in mutant and wild-type leaves (lanes 1 and 3, respectively). This result shows that the mutant RNA sample was not degraded and confirms the flower-specific nature of the *Ph6* transcript.

Figure 6C shows the comparable 28S rRNA signal given by the four RNA samples when the RNA gel blot was rehybridized with a wheat rDNA probe, confirming that the four lanes were loaded with approximately the same amount of RNA.

5 V. Isolation of *Ph6* DNA

Poly(A) RNA was isolated from total RNA from flower buds (Example IV above) and used to generate a cDNA library in the vector lambda ZapII (Stratagene). The SstI to BamHI fragment at the left hand side of Ac in Figure 2c was used to isolate pPet14-1 (containing *Ph6* cDNA), the sequence for which is given in Sequence ID No. 1. Sequencing was via dideoxy sequencing using a Promega fmol kit. Comparison of sequence information from the cDNA clone pPet14-1 (SEQ ID No. 3), and from the 4.7 and 6.8 Kb genomic clones described in Example IIB above was used to confirm that the Ac element had disrupted *Ph6* by insertion within the gene, and also to show that the *Ph6* gene is a transcriptional regulator.

15 VI. Suppression of endogenous *Ph6* gene in petunia plants transformed with *Ph6* constructs.

20 A. Constructs: The binary vectors p7575 and p7524 were designed and constructed to suppress expression of the wild-type *Ph6* gene in transgenic petunias. In these constructs, part of the *Ph6* gene was linked in the sense orientation to either the flower-specific petunia CHS-A promoter (Koes et al. (1989) *Plant Mol. Biol.* 12: 213-225) or the constitutive CaMV 35S promoter (Harpster et al. (1988) *Mol. Gen. Genet.* 212: 182-190) (see Figure 7). The part of the *Ph6* gene used in the constructs was a 1.36-kb SphI to SstI fragment of the 1.87-kb petunia *Ph6* cDNA, pPET14-1 (SEQ ID No. 3). This fragment includes the basic helix-loop-helix region (dark stippled box between PstI sites in Figure 7) and the acidic region (diagonally striped box to left of NdeI site in Figure 7). The gene fusions are transcribable but not translatable because of translational termination signals in all three reading frames at the 5' end of the *Ph6* sequence. In addition to the chimeric *Ph6* gene, these vectors contain a kanamycin resistance transformation marker (Beck et al. (1982) *Gene* 19: 327-336) between the left and right T-DNA borders.

For p7524, pPET14-1 was digested with SphI and SstI, and both sites were made blunt using T4 polymerase in the presence of all four deoxyribonucleotide triphosphates (dNTP's). The 1.36 Kb cDNA insert was gel purified through agarose and ligated into p2104-CABL (Harpster et al., *supra*), which had been digested with Xho I and Bam HI and treated with Klenow and dNTP's to create blunt ends. The resulting plasmid, p75119, contains 1.34 Kb of 35S promoter sequence, 60 bp of petunia CAB untranslated leader sequence, 1.36 Kb of Ph6 cDNA sequence in sense orientation, and 260 bp of *A. tumefaciens* nopaline synthetase transcriptional termination (NOS 3') sequence. The p35S-Ph6-NOS 3' insert was removed from p75119 by digestion with Eco RI and Hind III. The insert was gel purified and ligated into a binary vector (WTT2161) containing the neomycin phosphotransferase (NPT II) gene for transformation and selection of kanamycin-resistant plant tissue. The resulting plasmid was called p7524. The plasmid pWTT2161 contains DNA from the following sources:

1. *Escherichia coli* DNA in the pACYC184-derived part of the plasmid vector, the *lacZa* fragment of the β -galactosidase gene, the coding sequence of the *nptII* gene and the tetracycline resistance (*tet R*) gene.
2. *Pseudomonas aeruginosa* DNA in the pVSI-derived part of the plasmid vector.
3. *Agrobacterium tumefaciens* octopine strain DNA in the left and right border regions of the T-DNA and the polyadenylation signal of the octopine synthase gene.
4. *Agrobacterium tumefaciens* nopaline strain DNA in the promoter of the nopaline synthase gene.

For p7575, pPET14-1 was digested with Sph I and treated with T4 polymerase and dNTPs to create one blunt end and then digested with Sst I. The 1.36 Kb insert was gel purified and ligated in sense orientation into a pCHSA-NOS 3' cassette (WTT2145), which had been digested with Nco I, blunt-ended with Klenow and dNTPs, and then redigested with Sst I.

The resulting plasmid was called p7492. Plasmid pWTT2145 contains DNA from the following sources:

1. *Escherichia coli* DNA in the pUC119-derived part of the plasmid vector.
- 5 2. *Agrobacterium tumefaciens* nopaline strain DNA in the polyadenylation signal of the nopaline synthase gene.
3. *Petunia hybrida* DNA in the promoter of the CHSA gene.

10 Plasmid p7492 has approximately 800 bp of CHSA promoter sequence followed by the 1.36 Kb Ph6 cDNA sequence and 260 bp of NOS 3' sequence. The pCHSA-Ph6-Nos 3' gene fusion was released from the pUC-based plasmid in p7492 by digestion with Bgl II and Eco RI. It was then gel purified
15 and ligated into the Bam HI and Eco RI sites of a binary vector (WTT2161) for plant transformation and selection, to create p7575.

B. Transformation: Magenta-flowered Ph6 F1 petunia plants (174 plants) from the cross between lines V26 and M59
20 were transformed with the above constructs. The binary vectors p7575 and p7524 were introduced into V26xM59 petunia plants using the LBA 4404 strain of *Agrobacterium*, as described in Napoli et al., *Plant Cell* 2: 279-289 (1990), which is incorporated herein by reference. Transformants were
25 selected based on their ability to grow roots and shoots in the presence of kanamycin. Eight plants transformed with p7575 and seven transformed with p7524 were analyzed by Southern blots and confirmed to carry from one to three copies of the transgene. Plants (30) transformed with the binary
30 vector pWTT2161, containing only the kanamycin resistance marker but no petunia sequences, were used as controls.

C. Flower pigment phenotypes: Several transgenic plants exhibited a visible change in phenotype. The phenotypic change consisted in a shift in flower color from
35 magenta-red to blue as a consequence of an increase in corolla pH, as described below. The blue color faded with aging of the flower because of the presence in these plants of the Fa allele (see Example III).

As is generally observed in sense suppression experiments, only a fraction of the transgenic plants displayed a change in phenotype. More sense-suppressed plants were obtained with the p7524 construct than with the p7575 construct (20/94 vs. 1/80). The two constructs produced somewhat different phenotypes. In the altered plant transformed with the p7575 (CHS-A/Ph6) construct, the flowers were either uniformly blue or chimeras of blue and red tissue. After two to three days, the blue color faded to white due to the presence in these plants of the dominant fading factor *Fa*. The freshly opened flowers in sense suppressed plants that had been transformed with the p7524 (35S/Ph6) construct gave variable patterns of red and either white or pale blue tissue that quickly faded to white, often within the same day of opening. Again, the presence of the *Fa* factor can account for the fading of the pale blue color to white, although this fading appeared to happen much faster than in *ph6* mutant plants or plants transformed with the p7575 (CHS-A/Ph6) construct. No phenotypic changes were observed in the controls.

D. Corolla pH. The pH of corolla extracts was measured as described in de Vlaming et al., *Theor. Appl. Genet.* 66: 271-278 (1983), which is incorporated herein by reference (see Example III). At least 3 flowers per transformant for 21 transformants were assayed in duplicates. For both constructs, the pH of flower homogenates from plants which showed suppression of the *Ph6* gene was about 0.3 pH units higher than that of extracts from plants transformed with the same construct, but which did not show a phenotypic effect, i.e., which had magenta flowers similar to the control plants. This increase in pH was of the same magnitude as that produced by the *ph6* mutation. Chuck et al., *Plant Cell* 5: 371-378 (1993).

VII. OVEREXPRESSION OF PH6 GENE IN TOMATO

A. Constructs: A plasmid was constructed containing the full length cDNA of *Ph6* under control of the 35S promoter and with a 3' region from the NOS gene. A full length cDNA clone (2.2kb) was assembled by splicing together

fragments from two incomplete cDNA clones: a 0.77-Kb Pvu II-SacII fragment from one cDNA clone, containing an intact 5' end, and a 1.4-Kb SacII-XbaI fragment from another cDNA clone, containing an intact 3' end. The full-length cDNA was cloned
5 into a pBS (Stratagene)-based plasmid carrying the 35S/cabL promoter/leader and the NOS 3' end (Harpster et al., supra) to give plasmid p7692. An EcoRI-Hind III fragment containing the 35S/cabL/Ph6/NOS 3' construct was then cloned into the binary vector pWTT2161.

10 B. Transformation. Transformation of cotyledon pieces of Baxter Early Bush tomato with the plasmid containing Ph6 is carried out as follows. The control is pWTT2161 (binary without petunia Ph6 sequences). All manipulations are carried out under sterile conditions. A culture of
15 *Agrobacterium* LBA4404 containing the pWTT2161:Ph6 plasmid is grown for 24 hours in minimal A medium (10.5 g/l K_2PO_4 , 4.5 g/l KH_2PO_4 , 1 g/l $(NH_4)_2SO_4$, 0.5 g/l Na citrate. $2H_2O$, 0.25 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l glucose) at 28°C. Explants are excised from the mid-sections of 7-8 day old cotyledons of sterile grown *L.*
20 *esculentum* seedlings on germination medium (OMS plus 1% (w/v) sucrose and 0.8% (w/v) TC agar). OMS is MS salts and FeEDTA (Murashige and Skoog *Physiol. Plant* 15:473-497, 1962); B5 vitamins (Gamborg et al. *Exp. Cell Res.* 50:151-158, 1968; 3mM MES. The *Agrobacterium* culture is diluted in OMS plus 2%
25 (w/v) glucose to a final concentration of 5×10^5 /ml. The explants are submerged in the *Agrobacterium* suspension for 20-30 minutes and then placed on cocultivation medium (OMS plus 2% (w/v) sucrose, 0.05mg/l IAA, 1mg/l zeatin, 100 M acetosyringone and 0.8% TC agar) for 2 days at 25°C. The
30 explants are then placed on regeneration medium (OMS plus 3% sucrose, 0.05mg/l IAA, 0.5mg/l zeatin, 500mg/l carbenicillin, 200mg/l kanamycin and 0.8% TC agar) and cultured under high light fluence with an 8 hour dark period at 25°C. After about 10 days kanamycin resistant callus appears and then small
35 shoot buds by about 3 weeks. At about 5 weeks the healthy calli and shoots are transferred to fresh regeneration medium and within about 2 weeks transformed shoots emerge. The shoots are excised and transferred to rooting medium (OMS plus

1% sucrose, 500 mg/l carbenicillin, 150 mg/l kanamycin and 0.8% TC agar). Plants that successfully develop roots in this medium in about 6-10 days are transformants. These plants are transferred to non-selective rooting medium for 2 weeks to
5 check for residual Agrobacteria, then transplanted to soil.

C. Results. Transformants are screened for increased acidity of juice, both in terms of pH and titratable acidity, as compared to control. Normal variation in untransformed tomatoes is plus or minus 0.1 pH units.
10 Transformants with pH lowered by 0.2 pH units or more are chosen. Progeny are obtained by replanting of collected seed and stability/heritability of increased acidity is determined. Stable progeny are of use with respect to enhanced flavor and with respect to stability for processing purposes.

15

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims.

20

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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- (H) TELEFAX: (510) 547-2817
- (I) TELEX:

(ii) TITLE OF INVENTION: PH GENES AND THEIR USES

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: WO not yet assigned
- (B) FILING DATE: 15-APR-1994
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/049,282
- (B) FILING DATE: 16-APR-1993

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Bastian, Kevin L.
- (B) REGISTRATION NUMBER: 34,774
- (C) REFERENCE/DOCKET NUMBER: 12176-34-1PC

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (415) 543-9600
- (B) TELEFAX: (415) 543-5043

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1149 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1146

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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1 5 10 15	
TCC AAT ACA AAT ATG GAC TCT GAT TTC CAT ATG GTT GGA GTT AGC CAA	96
Ser Asn Thr Asn Met Asp Ser Asp Phe His Met Val Gly Val Ser Gln	
20 25 30	
GCC GAA AAC CCA GCT GAC TAC CAG CGC CAA GCT GAG TCA TTC AAA GCT	144
Ala Glu Asn Pro Ala Asp Tyr Gln Arg Gln Ala Glu Ser Phe Lys Ala	
35 40 45	
GAC ACT TCC ATT AGC TGG GCT CAT TTC CAA GAC CTT CCA CAT TTA CCA	192
Asp Thr Ser Ile Ser Trp Ala His Phe Gln Asp Leu Pro His Leu Pro	
50 55 60	
GGC GGC CCT AGT TAT GAT GAA TTA TCA CAA GAA GAC ACA CAT TAC TCT	240
Gly Gly Pro Ser Tyr Asp Glu Leu Ser Gln Glu Asp Thr His Tyr Ser	
65 70 75 80	
CAA ACA GTG TCG ACC ATT CTT GAA CAC CTC TCA AAC CAA AGC TCC AAA	288
Gln Thr Val Ser Thr Ile Leu Glu His Leu Ser Asn Gln Ser Ser Lys	
85 90 95	
TTT TCC TCT ACC ATA ATG GGC TGT ATT TCC CAA ACA ACC CAA TCT GCC	336
Phe Ser Ser Thr Ile Met Gly Cys Ile Ser Gln Thr Thr Gln Ser Ala	
100 105 110	
TTC ACA AGG TGG CCC AGC CCC AGC ACC ACC GTC TCC AGC CCA TTT CTT	384
Phe Thr Arg Trp Pro Ser Pro Ser Thr Thr Val Ser Ser Pro Phe Leu	
115 120 125	
GAC GGC GGC GCC ACC TCC GGC CAG TGG CTG CTC AAA AGC ATA CTA TTC	432
Asp Gly Gly Ala Thr Ser Gly Gln Trp Leu Leu Lys Ser Ile Leu Phe	
130 135 140	
TCT GTT CCA TTT CTT CAC ACT AAA TAC CAA ACT GCA GCT GAA GTT TCT	480
Ser Val Pro Phe Leu His Thr Lys Tyr Gln Thr Ala Ala Glu Val Ser	
145 150 155 160	
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Pro Lys Ser Arg Asp Ala Thr Thr Val Asp Ser Ser Thr Ala Ser Arg	
165 170 175	
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180 185 190	

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195 200 205	
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225 230 235 240	
CAG GAT CTT GAA GCT AGA GCC AAT CAG ACG GAG GCT ACG CTG CAG ACA Gln Asp Leu Glu Ala Arg Ala Asn Gln Thr Glu Ala Thr Leu Gln Thr	768
245 250 255	
AAG GAT ACA GGT ACT GTC AAG GTG TTG CAA GGA AGG GGT AAG AGG AGA Lys Asp Thr Gly Thr Val Lys Val Leu Gln Gly Arg Gly Lys Arg Arg	816
260 265 270	
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275 280 285	
GCA TCT TCG CCC TCA ACG ACA CAT GAA GAG GAG ATA GTG CAA GTA GAA Ala Ser Ser Pro Ser Thr Thr His Glu Glu Glu Ile Val Gln Val Glu	912
290 295 300	
GTA TCA ATT ATC GAG AGT GAT GCA CTG GTG GAG CTC AGG TGT CCA TAC Val Ser Ile Ile Glu Ser Asp Ala Leu Val Glu Leu Arg Cys Pro Tyr	960
305 310 315 320	
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325 330 335	
GTG GAG GTT GTA ACC ATT CAA TCA TCT CTT AAT AAT GGC AGC TTC TTT Val Glu Val Val Thr Ile Gln Ser Ser Leu Asn Asn Gly Ser Phe Phe	1056
340 345 350	
GCT GAG CTG AGA GCT AAG GTA AAA GAG AAT ATA TAT GGA AGG AAA GCC Ala Glu Leu Arg Ala Lys Val Lys Glu Asn Ile Tyr Gly Arg Lys Ala	1104
355 360 365	
AAG CAT TCT GGA AGT CAA GAA GTC AAT ACA CCA GTT AAT CCC Lys His Ser Gly Ser Gln Glu Val Asn Thr Pro Val Asn Pro	1146
370 375 380	
TAG	1149

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 382 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gln Leu Asp Met Ser Glu Ala Ile Arg Phe Gly Ser Pro Asp Asp Gly
 1 5 10 15
 Ser Asn Thr Asn Met Asp Ser Asp Phe His Met Val Gly Val Ser Gln
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 35 40 45
 Asp Thr Ser Ile Ser Trp Ala His Phe Gln Asp Leu Pro His Leu Pro
 50 55 60
 Gly Gly Pro Ser Tyr Asp Glu Leu Ser Gln Glu Asp Thr His Tyr Ser
 65 70 75 80
 Gln Thr Val Ser Thr Ile Leu Glu His Leu Ser Asn Gln Ser Ser Lys
 85 90 95
 Phe Ser Ser Thr Ile Met Gly Cys Ile Ser Gln Thr Thr Gln Ser Ala
 100 105 110
 Phe Thr Arg Trp Pro Ser Pro Ser Thr Thr Val Ser Ser Pro Phe Leu
 115 120 125
 Asp Gly Gly Ala Thr Ser Gly Gln Trp Leu Leu Lys Ser Ile Leu Phe
 130 135 140
 Ser Val Pro Phe Leu His Thr Lys Tyr Gln Thr Ala Ala Glu Val Ser
 145 150 155 160
 Pro Lys Ser Arg Asp Ala Thr Thr Val Asp Ser Ser Thr Ala Ser Arg
 165 170 175
 Phe Arg Lys Gly Cys Ser Ile Thr Gln Glu Glu Pro Ser Gly Asn His
 180 185 190
 Val Leu Ala Glu Arg Arg Arg Arg Glu Lys Leu Asn Glu Arg Phe Ile
 195 200 205
 Ile Leu Arg Ser Leu Val Pro Phe Val Thr Lys Met Asp Lys Ala Ser
 210 215 220
 Ile Leu Gly Asp Thr Ile Glu Tyr Val Lys Gln Leu Arg Lys Lys Val
 225 230 235 240
 Gln Asp Leu Glu Ala Arg Ala Asn Gln Thr Glu Ala Thr Leu Gln Thr
 245 250 255
 Lys Asp Thr Gly Thr Val Lys Val Leu Gln Gly Arg Gly Lys Arg Arg
 260 265 270
 Met Lys Ile Val Glu Gly Ser Val Gly Gly Gly Gln Ala Lys Ile Thr
 275 280 285
 Ala Ser Ser Pro Ser Thr Thr His Glu Glu Glu Ile Val Gln Val Glu
 290 295 300
 Val Ser Ile Ile Glu Ser Asp Ala Leu Val Glu Leu Arg Cys Pro Tyr

305				310					315					320		
Lys	Glu	Gly	Leu	Leu	Leu	Asp	Val	Ile	Glu	Met	Leu	Arg	Glu	Leu	Lys	
				325					330					335		
Val	Glu	Val	Val	Thr	Ile	Gln	Ser	Ser	Leu	Asn	Asn	Gly	Ser	Phe	Phe	
				340					345					350		
Ala	Glu	Leu	Arg	Ala	Lys	Val	Lys	Glu	Asn	Ile	Tyr	Gly	Arg	Lys	Ala	
				355					360					365		
Lys	His	Ser	Gly	Ser	Gln	Glu	Val	Asn	Thr	Pro	Val	Asn	Pro			
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1857 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 481..1632

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

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GAACTGGGGA CTACACAACG GATTCAAGAA GACATTGGAT TCATAAACCA TGTCAAGACT      180
TTCTTCATTG AGCAACAACC ACCTCTGCCG CCAAAGCCAG CCTTATCTGA GCACTCCACT      240
TCCAATCCCA CCACATTTTC AGAACTAAAC TTTTACTCCA GCAATACTCC ACCATCCGCT      300
GGCACTACTC CTGCGGATGA ACACGGTGGA GTAGCCGGAG ATGAAGACGA AGAAGATGAG      360
GATGAGGAGG ACGAAGACGA GGAACAAGAG GATGATGAAG AAGCCGAGTT GGACTCGGAT      420
AAGATAGCGG CTCAGGTTGG CCGGCTGAT GTTATAGCCG CGGCTGAGGC CAGTGAACCT      480

ATG CAG CTT GAT ATG TCT GAG GCA ATA CGG TTT GGG TCA CCG GAT GAC      528
Met Gln Leu Asp Met Ser Glu Ala Ile Arg Phe Gly Ser Pro Asp Asp
  1             5             10             15

GGC TCC AAT ACA AAT ATG GAC TCT GAT TTC CAT ATG GTT GGA GTT AGC      576
Gly Ser Asn Thr Asn Met Asp Ser Asp Phe His Met Val Gly Val Ser
             20             25             30

CAA GCC GAA AAC CCA GCT GAC TAC CAG CGC CAA GCT GAG TCA TTC AAA      624
Gln Ala Glu Asn Pro Ala Asp Tyr Gln Arg Gln Ala Glu Ser Phe Lys
             35             40             45

GCT GAC ACT TCC ATT AGC TGG GCT CAT TTC CAA GAC CTT CCA CAT TTA      672
Ala Asp Thr Ser Ile Ser Trp Ala His Phe Gln Asp Leu Pro His Leu
             50             55             60

CCA GGC GGC CCT AGT TAT GAT GAA TTA TCA CAA GAA GAC ACA CAT TAC      720
Pro Gly Gly Pro Ser Tyr Asp Glu Leu Ser Gln Glu Asp Thr His Tyr
             65             70             75             80

TCT CAA ACA GTG TCG ACC ATT CTT GAA CAC CTC TCA AAC CAA AGC TCC      768
Ser Gln Thr Val Ser Thr Ile Leu Glu His Leu Ser Asn Gln Ser Ser
             85             90             95

AAA TTT TCC TCT ACC ATA ATG GGC TGT ATT TCC CAA ACA ACC CAA TCT      816
Lys Phe Ser Ser Thr Ile Met Gly Cys Ile Ser Gln Thr Thr Gln Ser
             100            105            110

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Ala Phe Thr Arg Trp Pro Ser Pro Ser Thr Thr Val Ser Ser Pro Phe
             115            120            125

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TCT CCA AAG TCA CGT GAC GCT ACC ACT GTT GAT TCC TCC ACT GCA TCT Ser Pro Lys Ser Arg Asp Ala Thr Thr Val Asp Ser Ser Thr Ala Ser 165 170 175	1008
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AGA ATG AAG ATA GTG GAA GGA AGT GTT GGT GGA GGA CAG GCA AAG ATC Arg Met Lys Ile Val Glu Gly Ser Val Gly Gly Gly Gln Ala Lys Ile 275 280 285	1344
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TAC AAA GAA GGG TTG TTG TTA GAT GTA ATC GAA ATG CTA AGG GAA CTT Tyr Lys Glu Gly Leu Leu Leu Asp Val Ile Glu Met Leu Arg Glu Leu 325 330 335	1488
AAA GTG GAG GTT GTA ACC ATT CAA TCA TCT CTT AAT AAT GGC AGC TTC Lys Val Glu Val Val Thr Ile Gln Ser Ser Leu Asn Asn Gly Ser Phe 340 345 350	1536
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GCC AAG CAT TCT GGA AGT CAA GAA GTC AAT ACA CCA GTT AAT CCC TAGAGTTTAA 1639 Ala Lys His Ser Gly Ser Gln Glu Val Asn Thr Pro Val Asn Pro 370 375 380	
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WO 94/23561

PCT/US94/04173

44

TTTGGGCACG CCAACGAGT AAATAGAATA GAATAAAGGA TTAGATTATG TATATGGACA 1819
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(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 383 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Gln Ala Glu Asn Pro Ala Asp Tyr Gln Arg Gln Ala Glu Ser Phe Lys
          35           40           45
Ala Asp Thr Ser Ile Ser Trp Ala His Phe Gln Asp Leu Pro His Leu
          50           55           60
Pro Gly Gly Pro Ser Tyr Asp Glu Leu Ser Gln Glu Asp Thr His Tyr
          65           70           75           80
Ser Gln Thr Val Ser Thr Ile Leu Glu His Leu Ser Asn Gln Ser Ser
          85           90           95
Lys Phe Ser Ser Thr Ile Met Gly Cys Ile Ser Gln Thr Thr Gln Ser
          100          105          110
Ala Phe Thr Arg Trp Pro Ser Pro Ser Thr Thr Val Ser Ser Pro Phe
          115          120          125
Leu Asp Gly Gly Ala Thr Ser Gly Gln Trp Leu Leu Lys Ser Ile Leu
          130          135          140
Phe Ser Val Pro Phe Leu His Thr Lys Tyr Gln Thr Ala Ala Glu Val
          145          150          155          160
Ser Pro Lys Ser Arg Asp Ala Thr Thr Val Asp Ser Ser Thr Ala Ser
          165          170          175
Arg Phe Arg Lys Gly Cys Ser Ile Thr Gln Glu Glu Pro Ser Gly Asn
          180          185          190
His Val Leu Ala Glu Arg Arg Arg Arg Glu Lys Leu Asn Glu Arg Phe
          195          200          205
Ile Ile Leu Arg Ser Leu Val Pro Phe Val Thr Lys Met Asp Lys Ala
          210          215          220
Ser Ile Leu Gly Asp Thr Ile Glu Tyr Val Lys Gln Leu Arg Lys Lys
          225          230          235          240
Val Gln Asp Leu Glu Ala Arg Ala Asn Gln Thr Glu Ala Thr Leu Gln
          245          250          255
Thr Lys Asp Thr Gly Thr Val Lys Val Leu Gln Gly Arg Gly Lys Arg
          260          265          270
Arg Met Lys Ile Val Glu Gly Ser Val Gly Gly Gly Gln Ala Lys Ile
          275          280          285
Thr Ala Ser Ser Pro Ser Thr Thr His Glu Glu Glu Ile Val Gln Val
          290          295          300

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Glu Val Ser Ile Ile Glu Ser Asp Ala Leu Val Glu Leu Arg Cys Pro
305 310 315 320
Tyr Lys Glu Gly Leu Leu Leu Asp Val Ile Glu Met Leu Arg Glu Leu
325 330 335
Lys Val Glu Val Val Thr Ile Gln Ser Ser Leu Asn Asn Gly Ser Phe
340 345 350
Phe Ala Glu Leu Arg Ala Lys Val Lys Glu Asn Ile Tyr Gly Arg Lys
355 360 365
Ala Lys His Ser Gly Ser Gln Glu Val Asn Thr Pro Val Asn Pro
370 375 380

WHAT IS CLAIMED IS:

1. An isolated DNA construct comprising a polynucleotide sequence of at least about 30 nucleotides from a Ph gene.
2. The DNA construct of claim 1, wherein the Ph gene controls expression of a gene encoding a protein which regulates vacuolar pH.
3. The DNA construct of claim 2, wherein the Ph gene encodes a protein having a helix-loop-helix motif.
4. The DNA construct of claim 1, wherein the Ph gene is from petunia.
5. The DNA construct of claim 1, wherein the Ph gene is Ph6.
6. The DNA construct of claim 1, wherein the polynucleotide sequence is substantially identical to a sequence of least about 30 nucleotides from SEQ. ID. No. 1.
7. The DNA construct of claim 1, wherein the polynucleotide sequence is substantially identical to SEQ. ID. No. 1.
8. The DNA construct of claim 1, wherein the polynucleotide sequence is a full length Ph gene.
9. The DNA construct of claim 1, further comprising a promoter operably linked to the polynucleotide sequence.
10. The DNA construct of claim 9, wherein the polynucleotide sequence is linked to the promoter in an antisense orientation.

11. The DNA construct of claim 9, wherein the promoter is a plant promoter.

12. The DNA construct of claim 11, wherein the promoter is a fruit-specific promoter.

13. The DNA construct of claim 11, wherein the promoter is a flower-specific promoter.

14. A DNA construct comprising a polynucleotide sequence of at least about 30 nucleotides from a gene which confers one or more of the following characteristics on a plant bearing a mutant form of the gene:

- 1) increased vacuolar pH;
- 2) alteration of anthocyanin pigments, thereby producing flowers with a bluish color;
- 3) flower color fades with aging; and
- 4) shrivelled or irregularly shaped seed having less pigment than a wild-type seed.

15. The DNA construct of claim 1, wherein the *Ph* gene is *Ph6* from *Petunia*.

16. A transgenic plant comprising a recombinant expression cassette comprising a plant promoter operably linked to a polynucleotide sequence of at least about 30 nucleotides from a *Ph* gene.

17. The transgenic plant of claim 16, wherein the plant promoter is a heterologous promoter.

18. The transgenic plant of claim 16, wherein the polynucleotide sequence is linked to the promoter in an antisense orientation.

19. The transgenic plant of claim 16, wherein the plant is *petunia*.

20. The transgenic plant of claim 16, wherein the *Ph* gene regulates expression of genes encoding proteins which affect vacuolar pH.

21. The transgenic plant of claim 16, wherein the *Ph* gene encodes a protein having a helix-loop-helix motif.

22. The transgenic plant of claim 16, wherein the *Ph* gene is from petunia.

23. The transgenic plant of claim 16, wherein the *Ph* gene is *Ph6*.

24. The transgenic plant of claim 16, wherein the polynucleotide sequence is substantially identical to a sequence of at least 30 nucleotides from SEQ. ID. No. 1.

25. A method of altering vacuolar pH in a plant, the method comprising:

introducing into plant tissue a recombinant expression cassette comprising a plant promoter operably linked to a polynucleotide sequence from a *Ph* gene;
regenerating the plant tissue into a whole plant, whereby the regenerated plant transcribes the polynucleotide sequence; and
selecting plants having altered vacuolar pH.

26. The method of claim 25, wherein the plant tissue is from petunia, rose or tomato.

27. The method of claim 25, wherein the recombinant expression cassette is introduced into the plant tissue using *Agrobacterium*.

28. The method of claim 25, wherein the polynucleotide sequence is linked to the promoter in an antisense orientation.

29. The method of claim 25, wherein the polynucleotide sequence is linked to the promoter in a sense orientation.

30. The method of claim 25, wherein the polynucleotide sequence is substantially identical to SEQ. ID. No. 1.

31. The method of claim 25, wherein the promoter is a fruit-specific promoter.

32. The method of claim 25, wherein the promoter is a flower-specific promoter.

33. A method of isolating a *Ph* gene from a plant, the method comprising probing a DNA library prepared from the plant with oligonucleotide probes comprising a polynucleotide sequence from an isolated *Ph* gene.

34. The method of claim 33, wherein the DNA library comprises cDNA.

35. The method of claim 33, wherein the isolated *Ph* gene is *Ph6* from *Petunia*.

36. A method of isolating a *Ph* gene from a plant, the method comprising:

transforming plants with a DNA construct comprising a transposon;

assaying the plants for increased vacuolar pH resulting from the transposon excising from the DNA construct and inserting in the *Ph* gene;

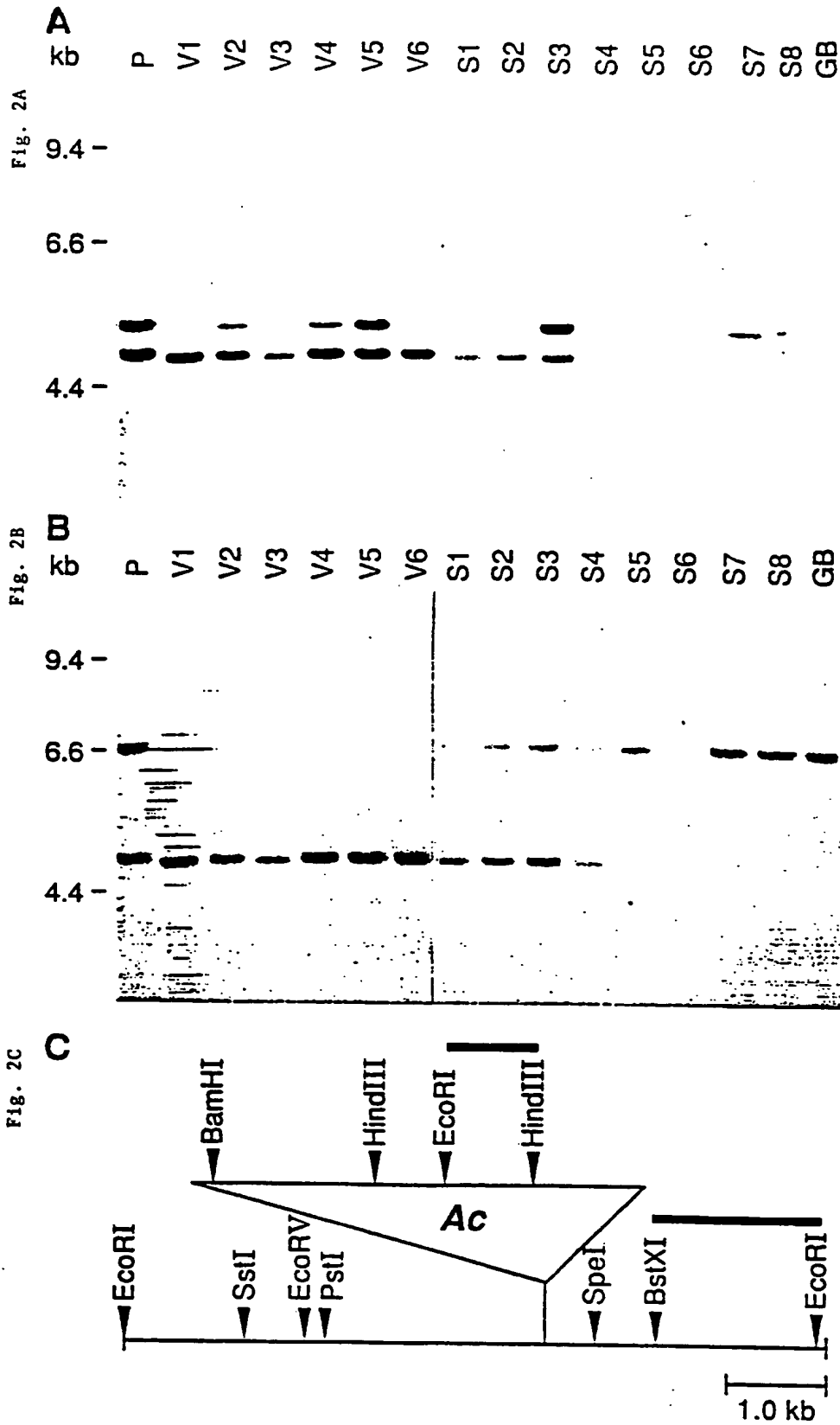
selecting those plants having increased vacuolar pH; and

isolating the *Ph* gene from the selected plants.

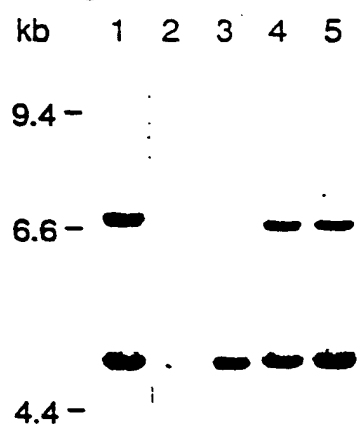
37. The method of claim 36, wherein the transposon is an *Ac* transposon.

38. The method of claim 36, wherein the transposon is inserted in a streptomycin resistance gene such that the gene is inoperable, whereby plants in which the transposon has excised from the gene are identified by the ability to grow on streptomycin.

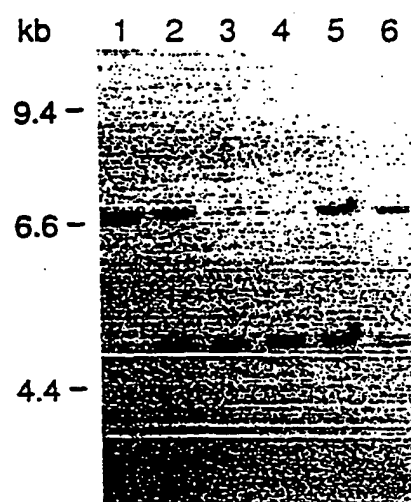




A Fig. 3A



B Fig. 3B



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Fig. 4

Fig. 5A



Fig. 5B

Fig. 6A

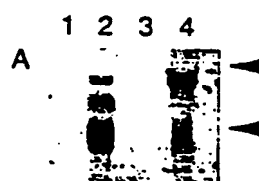


Fig. 6B

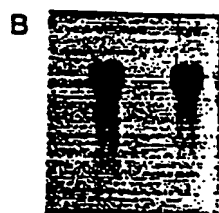


Fig. 6C



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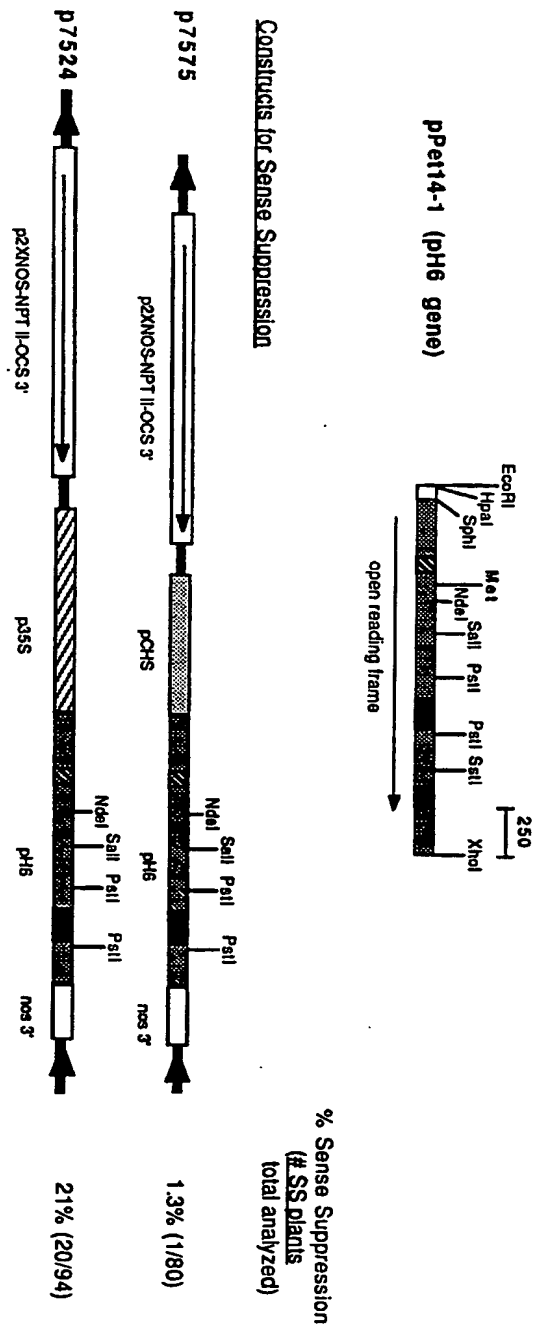


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04173

A. CLASSIFICATION OF SUBJECT MATTER IPC(5) : A01H 5/00; C12N 15/00 US CL : 435/172.3; 800/205, 255; 935/18 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/172.3; 800/205, 255; 935/18 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THEORETICAL AND APPLIED GENETICS, Volume 66, issued 1983, "Genes affecting flower colour and pH of flower limb homogenates in <i>Petunia hybrida</i> ", pages 271-278, see pages 271, Summary; 273, column 1, lines 1-27; page 277, column 2, lines 2-3.	1-8, 14-15, 20-24, 30, 35
Y	MONOGRAPHS ON THEORETICAL AND APPLIED GENETICS, Volume 9, issued 1984, "Inheritance and Biochemistry of Pigments", pages 49-67, see pages 63, paragraph 1; 64, Table 6.3.	1-8, 14-15, 20-24, 30, 35
Y	DEVELOPMENTAL GENETICS, Volume 10, issued 1989, "Gene Tagging in <i>Petunia hybrida</i> Using Homologous and Heterologous Transposable Elements", pages 561-568, see page 567, column 1, paragraphs 3-4 and column 2.	33-38
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family	
Date of the actual completion of the international search 13 JULY 1994		Date of mailing of the international search report JUL 21 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer <i>E. Veitenheimer</i> ERICH E. VEITENHEIMER Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04173

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PLANT MOLECULAR BIOLOGY, Volume 16, issued 1991, "The use of transgenic plants to understand transposition mechanisms and to develop transposon tagging strategies", pages 449-461, see pages 449, columns 1-2; 456, column 2, last paragraph; 457, columns 1-2; 458, columns 1-2; and 459, column 1.	33-38
Y	THE PLANT CELL, Volume 2, issued November 1990, "Molecular Characterization of a Nonautonomous Transposable Element (<i>dTph1</i>) of Petunia", pages 1121-1128, see entire article.	1-8, 14-15, 20-24, 30, 33-38
Y	PLANT MOLECULAR BIOLOGY, Volume 13, issued 1989, "A comparative study of Tam3 and Ac transposition in transgenic tobacco and petunia plants", pages 189-201, see entire article.	9, 11-13, 16-17, 19, 25-27, 29, 31-32
Y	NATURE, Volume 346, issued 19 July 1990, "Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants", pages 284-287, see entire article.	10, 18, 28